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Identification of genes differentially expressed during the interaction between the plant symbiont *Suillus luteus* and two plant pathogenic allopatric *Heterobasidion* species

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Abstract

The effects of biological invasions by non-native species have been widely studied in terms of environmental, economic, and human health impacts. However, little is known on the consequences that non-native plant pathogens may determine on host plant symbionts, such as ectomycorrhizal (ECM) fungi. In this study, interactions between *Suillus luteus,* an ECM fungus of pine trees, and the allopatrically differentiated fungal pathogens of pines *Heterobasidion irregulare* and *H. annosum* were investigated in dual culture by morphological and gene expression analyses. Growth of *S. luteus* was inhibited by the both *Heterobasidion* species, but based on statistical analysis growth inhibition was due to the isolate rather than to the species. The expression analysis on genes related to cell wall hydrolytic enzymes and hydrophobins, putatively involved in the fungus-fungus interaction, allowed to identify significantly up- and down- regulated genes both in the symbiont and in the pathogens. Based on the transcript analysis, it was not possible to distinguish the impact of the two pathogenic species on the ECM fungus. The only exception was a *S. luteus* gene coding for a putative chitinase (*SlGH18_8356*) that was found to be differentially regulated during interaction with *H. irregulare* compared to *H. annosum.*

Keywords: ectomycorrhizal fungus, fungal pathogen, dual culture, gene expression, cell wall, phylogeny

Introduction

Non-native invasive organisms stand among the main elements of global change and are playing a role in the biodiversity loss, ecosystem degradation, and impairment of ecosystem services (Pysek and Richardson 2010). In the worst case scenario, invasive organisms can also determine the extinction of native species (Lövei 1997). The effects of biological invasions have been mostly studied in terms of environmental, economic, and human health impacts (Keller et al. 2011).

Heterobasidion irregulare Garbel. & Otrosina is an invasive fungal pathogen of pines, introduced from North America to Italy during World War II (Gonthier et al. 2004). Once introduced, it has colonized pine and oak stands along 103 km of coastline west of Rome (Gonthier et al. 2004; Gonthier et al. 2007; Gonthier et al. 2012; Garbelotto et al. 2013). Its sister species *H. annosum* (Fr.) Bref. is also present in the coastal pine stands west of Rome, but only sporadically (Gonthier et al. 2007). *H. irregulare* and *H. annosum* have been compared in terms of pathogenicity on a range of pine species (Garbelotto et al. 2010; Pollastrini et al. 2015). Transcriptomic approaches, including qRT-PCR and microarray experiments, have been carried out on both species in order to better characterize the expression of several gene categories, focusing on saprobic growth and substrate specificity (Yakovlev et al. 2012; Raffaello et al. 2014; Baccelli et al. 2015). However, little is known on the possible and differential effects that these fungi may have on other components of native ecosystems, including host plant symbionts.

It is known that some ectomycorrhizal (ECM) fungi grown in dual culture with saprotrophic fungi may affect the physiology of the latter. *Laccaria laccata* (Scop.) Cooke, for example, has been reported to cause protoplast release and to penetrate the hyphae of soil saprotrophic fungi such as *Mucor hiemalis* Wehmer (Werner and Zadworny 2003), *Trichoderma harzianum* Rifai (Zadworny et al. 2004), *T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx (Werner et al. 2002). Other ECM fungi showed antagonistic activities (*i.e., Amanita muscaria* (L.) Lam*, Suillus bovinus* (L.) Roussel, *S. luteus* (L.) Roussel) against the fungi listed above (Mucha et al. 2006). Moreover, there is evidence that ECM fungi may display antibiotic activity both against saprotrophic and pathogenic fungi (Mucha et al. 2009), the ability to inhibit the growth of root pathogens (Cervinkova 1989; Lei et al. 1995; Mohan et al. 2015) and to modulate mycotoxin gene expression (Ismail et al. 2011).

By using the dual culture technique, Adomas et al. (2006) were able to investigate, under a molecular and physiological perspective, the interaction between *Heterobasidion parviporum* Niemelä & Korhonen and the saprotrophic basidiomycete *Phlebiopsis gigantea* (Fr.) Jülich, and Iakovlev et al. (2004) identified genes

differentially expressed in the interaction between *H. annosum* and the basidiomycete *Physisporinus sanguinolentus (*Alb. & Schwein.) Pilát*.*

During interaction in dual culture, a crucial role is played by the contact of the fungal cell walls. Fungal cell wall is made up of proteins and polysaccharides, mainly glucan and chitin, and its formation and remodeling requires the concerted action of several gene products that include several enzymes intimately associated with the fungal cell wall (Bowman and Free 2006). However, the cell wall can be subjected to degradation when exposed to hydrolytic enzymes produced by other organisms (Sivan and Chet 1989). In addition, filamentous fungal cell wall contains proteins playing a role in the interaction with the environment and in aggregation/adhesion events. Hydropobins are such small secreted proteins that are highly surface-active (Wösten and Scholtmeijer 2015). The dual culture approach has been employed to investigate the interaction between native microbes or to determine the effects of mycorrhizal fungi on other fungi (see references listed above). On the contrary, in this work, we tested the hypothesis that a non-native invasive fungal pathogen may have greater effects on ECM fungi than native fungal pathogens using *H. irregulare/H. annosum* and the ECM fungus *S. luteus* as a model system. Secondarily, the effects of the symbiont on the pathogens were also investigated. Our specific aims were: 1) to test if the interaction between a native symbiont and a invasive/native pathogen might affect fungal growth and morphology; 2) to verify if changes may occur in gene expression, focusing on genes encoding proteins related to cell wall degradation/modification and cell-to-cell adhesion (*i.e.,* hydrophobins); and 3) to test if differential gene expression might allow to distinguish the impact of the invasive *H. irregulare* compared to the native *H. annosum* may have on the native symbiont *S. luteus.*

Material and methods

Fungal isolates and culture maintenance

S. luteus LMSL8 was isolated from a basidiocarp, collected in Lommel Sahara, Limburg, Belgium. The fungus was grown in Petri dishes filled with Fries and Munzenberger medium (pH 4.8) (Fries 1978) and the cultures were kept in a dark room at 25°C. In one liter of distilled water, the medium contains: 10 g agar, 6 g glucose, 1 g di-ammonium tartrate, 30 mg KH_2PO_4, 0.1 g $MgSO_4$ $7H_2O$, 20 mg NaCl, 0.26 mg $CaCl_2$ $2H_2O$, 0.1 g KCl, 20 mg $FeCl_3$ $6H_2O$, 8.5 mg $MnSO_4$ H_2O, 1.25 mg $CuSO_4$ $5H_2O$, 0.2 mg $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 5.75 mg $ZnSO_4$ $7H_2O$, 15 mg H_3BO_3 and 10 ml of vitamin stock solution. Stock solution contains: 56 µM myo-inositol, 0.1 µM biotin, 0.5 µM pyridoxine, 0.3 µM riboflavin, 0.8 µM nicotinamide, 0.7 µM p-aminobenzoic

107 acid, 0.3 µM thiamine, 0.2 µM Ca-pantothenate. Each 7 days the culture was sub-cultured and refreshed
108 according to Kohler et al. 2015.
109 Three heterokaryotic (ploidy: n+n) isolates of *H. irregulare* and three heterokaryotic isolates of *H. annosum*
110 (Table 1), randomly selected among those available in the culture collection of the DISAFA at the University
111 of Turin were used. Isolates were deposited at the *Mycotheca Universitatis Taurinensis* (MUT) with the
112 accession numbers listed in the Table 1. All the isolates had been previously collected in Italy and identified
113 at the species level through taxon specific primers and AFLPs (Gonthier et al. 2007; Gonthier and Garbelotto
114 2011). The isolates were long term-stored at 4°C in mycological tubes (180 mm long x 18 mm diameter)
115 containing Malt Extract Agar (MEA: 31.3 g malt extract agar, 1L distilled water). Ten days before starting the
116 dual culture, all isolates were sub-cultured in new Petri dishes containing MEA. Mycelial plugs (0.5 mm
117 diameter) taken from the edge of actively growing colonies were used in the dual culture experiments
118 (Giordano et al. 2014).
119
120 **Comparative studies on antagonistic effects between *S. luteus* and *Heterobasidion* spp.**
121 Dual culture technique was used in comparative studies on antagonistic effects between *S. luteus* and
122 *Heterobasidion* spp. Mycelial plugs 5 mm in diameter were removed with a cork borer from actively growing
123 cultures (7 days old) and were paired, with the mycelial surface down, about 15 mm apart, 30 mm from the
124 edge of a 90 mm Petri dish containing Fries and Munzenberger agar. *S. luteus* and *Heterobasidion* spp.
125 isolates were paired in all possible combinations (dual cultures). For each *S. luteus*-*Heterobasidion* spp.
126 combination ten replicates were used and additionally ten replicates consisting of only one mycelial plug of
127 each fungal isolate were maintained as controls (pure cultures). All the Petri dishes were incubated at 25°C
128 in the dark.
129 Internal radial growth of the colonies in dual and pure cultures was measured every 48 hours under a
130 dissecting microscope and the growth rate of all the isolates, expressed in mm of colonization per day, was
131 calculated. Measurements were completed at the time that a corresponding control culture reached the
132 opposite edge of the Petri dish.
133 The mycelial interactions were scored using a system of classification according to Holdenrieder (1984): A)
134 the two colonies coexist side by side without interacting; B) formation of a mycelium-free inhibition zone
135 between the two mycelia, which stop expanding; C) *S. luteus* completely overgrows *Heterobasidion* spp.; D)
136 *S. luteus* partly overgrows *Heterobasidion* spp.; E) *Heterobasidion* spp. completely overgrows *S. luteus;* F)
137 *Heterobasidion* spp. partly overgrows *S. luteus.*

At the end of the experiment, the final fungal growth was determined in mm^2 of the mycelium surface and the zone of inhibition was determined. Areas colonized by the fungi were measured with a planimeter, as previously described (Nicolotti et al. 1999). Final mycelium surfaces of *H. irregulare*, *H. annosum* and *S. luteus* isolates in dual cultures were compared to those of control cultures using the Wilcoxon test.

For each isolate in dual culture, the inhibition growth rate (IGR in %) of average mycelium surface relative to the control was calculated as follows:

$$\mathrm{IGR\%} = \frac{\mathrm{mm^2\ control} - \mathrm{mm^2\ dual}}{\mathrm{mm^2\ control}} \cdot 100$$

where: mm^2 control = average mycelium surface in control cultures, mm^2 dual = average mycelium surface in dual cultures.

Since a viability test by using specific dye like FUN-1 (Millard et al. 1997; Lass-Flörl et al. 2001) was not feasible in our model system because formation of clamps in *Heterobasidion* spp. is infrequent, thus hampering to easily distinguish hyphae of *Heterobasidion* spp. from hyphae of the clampless *S. luteus*, a growth recovery test was performed. After 3 weeks of incubation, in Petri dishes where *S. luteus* had been over-grown by *Heterobasidion* spp. or *vice versa*, mycelial plugs were taken and transferred onto Fries and Munzenberger agar to test growth recovery of the fungus. Lack of growth from these mycelial plugs after 2 weeks' incubation at 25°C in the dark were regarded as *S. luteus* had possibly been killed or had at least been growth-arrested by *Heterobasidion* spp. or *vice versa*.

The IGRs in % of *S. luteus* and *Heterobasidion* spp. were logit-transformed (*i.e.,* logit inhibition growth rates - LIGRs) and tested for normality with the Shapiro-Wilk test, with a cut-off value set to 0.05 (Crawley 2013). Markov Chain Monte Carlo Generalized Linear Mixed Models (MCMC GLMMs) were fitted to test the effects of *Heterobasidion* spp. (*i.e.,* fixed factor) and isolates (*i.e.,* nested random factor) on the observed LIGRs. For both *S. luteus* and *Heterobasidion* spp. the MCMC GLMMs included the null model (M0), the two models with either fixed (M1) or random factors (M2) and the model with both these factors (M3). All MCMC GLMMs were fitted in R environment to estimate the β and Z coefficients, for fixed and random factors respectively, with the associated 95% confidence interval (CI95%) and p value (PMCMC) as described by Giordano et al. (2014), with the exception of the selected link function (*i.e.,* identity) and family distribution for the error term (*i.e.,* Gaussian) (Hadfield 2010; Kéry 2010). The Deviance Information Criterion (DIC) was calculated for

each MCMC GLMM and the minimum DIC method was used to select the best model displaying at least one significant coefficient other than the intercept (Berg et al. 2004; Crawley 2013).

Primer design for gene expression analysis

Taking advantage of the recently released genome sequence of *H. irregulare* (Olson et al. 2012) and *S. luteus* (Kohler et al 2015), we have identified genes related to chitinases (Glycoside Hydrolase family 18 - GH18), β-N-acetylglucosaminidases (Glycoside Hydrolase family 20 - GH20), endo-β-N-acetylglucosaminidases (Glycoside Hydrolase family 85 - GH85) and hydrophobins.

For *S. luteus*, 22 qRT-PCR primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), considering the specific gene categories listed above. Full-length sequences of these gene categories were obtained from the *S. luteus* genome (http://genome.jgi-psf.org/Suilu1/Suilu1.home.html).

For *H. irregulare*, 17 primer pairs were designed by using Primer3Plus to target genes belonging to the same categories as above (*i.e.,* GH18, GH20, GH85, hydrophobins). Full-length sequences of candidate genes were obtained from the available *H. irregulare* genome (http://genome.jgi-psf.org/Hetan2/Hetan2.home.html). Candidate genes of *H. irregulare* were selected on the basis of representativeness within each sub-class and of their nucleotide identity to sequences of *H. annosum* (Sillo and Gonthier, unpublished). Before qRT-PCR, all the primers were tested *in silico* on Primer-BLAST and *in vitro* in PCR reactions on genomic DNA extracted from all the three species with the Dneasy Plant mini kit (Qiagen, Valencia, CA, USA) in order to verify the absence of cross amplification.

Sequence and bioinformatic analyses

The protein sequences deduced from the gene sequences were used for extensive database searches for both homologous sequences and sequences that were closely related phylogenetically, *i.e.,* pathogenic, saprotrophic and mycorrhizal Basidiomycota and Ascomycota sequences. A multiple protein alignment was built using the MUSCLE software inside Mega version 6.

Phylogenetic trees were constructed using the Neighbor joining (NJ) method, with the Mega software version 6 (Tamura et al. 2013). Bootstrap analyses were carried out on the basis of 1,000 re-samplings of the sequence alignment.

Sequences were further analyzed using SignalP 4.1 (Peterson et al. 2011) to ensure that they also contain a signal peptide.

198

199 **RNA extraction and cDNA conversion**

200 The same approach as above consisting of dual and pure cultures was used to harvest the mycelium for the
201 gene expression studies. The only differences were that each system was produced in triplicate and that
202 before fungal inoculation the agar surface was covered with a cellophane membrane in order to facilitate the
203 harvest of the mycelia. The cellophane membranes were cut to fit the size of Petri dishes and autoclaved for
204 20 minutes. The mycelium was harvested from pure cultures of both the symbiont and the pathogenic
205 isolates as well as from the interface and the outside of mycelia in dual culture as shown in Fig. 1. Outside
206 mycelia were considered as previously done by Adomas et al. (2006). The tubes containing the frozen
207 mycelium were put in a freeze dry machine overnight at -65°C before the RNA extraction. The RNA was
208 extracted using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. After
209 extraction, the RNA was cleaned of DNA using Promega DNase (RQ1 RNase-Free DNase, Promega Corp.,
210 Madison, WI, USA) and measured using a NanoDrop (Thermo Scientific, Hudson, NH, USA). The absence
211 of genomic DNA was verified through one-step retrotranscription PCR (One-Step RT-PCR, Qiagen) using
212 the primers for the housekeeping gene*, i.e.,* respectively the elongation factor 1α for *S. luteus*
213 (*Sl_699467*_EF1A; Table S1A) and *Tryp Metab* (Protein ID: 43087) for *Heterobasidion* spp. (Raffaello and
214 Asiebgu 2013, Table S1B).
215 Five hundred ng of total RNA was used for each sample to synthesize the cDNA, according to the
216 SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) procedure.
217

218 **Gene expression analysis**

219 Quantitative RT-PCR was carried out with the Connect™ Real-Time PCR Detection System (Bio-Rad
220 Laboratories, Hercules, CA, USA). Each PCR reaction was conducted on a total volume of 10 µl, containing
221 1 µl diluted cDNA (dilution 1:3), 5 µl SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad
222 Laboratories) and 2 µl of each primer (3 µM), using a 96 well plate. Primer sequences with their optimal
223 annealing temperature are listed in Tables S1A-B. The following PCR program, which includes the
224 calculation of a melting curve, was used: 95°C for 30 s, 40 cycles of 95°C for 10 s, the optimal annealing
225 temperature for 30 s, ramp from 65°C to 93°C with a temperature increment of 0.5°C and a read plate every
226 2 s. All the reactions were performed for three biological and three technical replicates. The baseline range
227 and Ct values were automatically calculated using the Bio-Rad CFX Manager software. In order to compare

the data from different PCR runs or cDNA samples, the Ct values of all the genes were normalized to the Ct value of the housekeeping gene.

The candidate gene expression was normalized to that of the housekeeping gene by subtracting the Ct value of the housekeeping gene from the Ct value of the candidate gene efficiency correction, from equation $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001), where ΔΔCt represents the ΔCt sample − ΔCt control (pure culture). Statistical analyses were carried out using Rest 2009, version 2.0.13, considering 0.05 as the *p* value. Only significant expression values were considered. A custom R script was used to visualize gene expression values as HeatMaps. This representation of the transcript levels coupled to a hierarchical clustering was performed in order to group genes with similar expression profiles. In addition, an unpaired *t-test* was carried out to determine differentially regulated genes in *H. irregulare* compared to *H. annosum*, as well as *S. luteus* genes differentially regulated during interaction with the invasive and the native *Heterobasidion* spp. Eventually, principal component analysis (PCA) of the whole gene expression data set was run using MeV v4.9 (http://www.tm4.org/mev.html).

Results

Comparative studies on antagonistic effects between *S. luteus* and *Heterobasidion* spp.

Pure cultures of all *Heterobasidion* spp. isolates reached the opposite edge of Petri dish after 9 days of incubation, while cultures of *S. luteus* did not.

The growth of *S. luteus* was significantly inhibited by all *Heterobasidion* spp. isolates (*S. luteus* IGR > 70%, $P < 0.05$). The only exception was observed between *S. luteus* and *H. annosum* 137OC-142OH isolate (*S. luteus* IGR 49%) (Fig. 2).

No inhibition zone was observed in the dual cultures. *Heterobasidion* spp. isolates always completely overgrew *S. luteus.* In the growth recovery test, after 2 weeks' incubation, *S. luteus* growth was never observed.

The LIGRs of *S. luteus* and *Heterobasidion* spp. were normally distributed based on the Shapiro-Wilk test ($P > 0.05$). The DIC values observed in the MCMC GLMMs ranged from 79.043 to 112.547 for *S. luteus* LIGR and from 67.402 to 104.876 for *Heterobasidion* spp. LIGR, with the largest values achieved by M0 and M1 in both cases. M2 was the model displaying the lowest DIC and at least one significant coefficient ($P < 0.05$), other than the intercept, for both *S. luteus* and *Heterobasidion* spp. LIGRs. In the case of *S. luteus* LIGR, the *Z* coefficients were significant ($P < 0.05$) in M2 for the combination composed of *H. annosum* 137OC-142OH

and *S. luteus*, in the case of *Heterobasidion* spp. LIGR for combinations including *S. luteus* and *H. irregulare* MUT00001151, *H. irregulare* MUT00003560 and *H. annosum* 137OC-142OH, respectively. The *β* coefficient for the fixed factor was significant ($P < 0.05$) only in the M1 of *S. luteus* LIGR (Table S2).

Sequence and bioinformatic analysis

Eight GH18, *i.e.,* putative chitinases, were considered in *S. luteus*. As revealed by means of phylogenetic analysis, three belonged to B subgroup, while the others to A subgroup, *sensu* Gruber et al. (2011). Six GH18 were analyzed in *H. irregulare*: four belonged to the subgroup A, whereas the remaining belonged to subgroup B (Fig. 3). All the sequences, except SlGH18_79518, HiGH18_11431 and HiGH18_3306, showed the presence of a secretory signal peptide.

Two genes coding for putative GH20, *i.e.,* β-N-acetylglucosaminidases, as well as two coding for putative GH85, *i.e.,* endo-β-N-acetylglucosaminidases, were found in the *S. luteus* genome. Members of the second family in this ECM fungus did not have the signal peptide. In *H. irregulare* there were four genes coding for putative GH20 and one for a putative GH85.

Nine hydrophobins, with a putative role in the adhesion and in the interaction with the environment, were present in *S. luteus* and eight in *H. irregulare,* all belonging to class I hydrophobins. Multiple protein sequence alignment revealed the presence of three main subgroups of hydrophobins in *S. luteus*. There was also a clear distinction between the hydrophobins of the two fungi; the *Heterobasidion* spp. sequences were divided in three clusters far from *S. luteus* sequences, as revealed by means of phylogenetic analysis (Fig. 4). Moreover, *S. luteus* sequences were grouped together with hydrophobins of other symbiont basidiomycetes, such as *Laccaria bicolor* and *Paxillus involutus* (Batsch) Fr. All the putative *S. luteus* hydrophobin sequences had a predicted signal peptide and a hydrophobin/HYDRO domain was recognized by means of InterProScan, except in SlHyd_804366. All the putative *H. irregulare* hydrophobins harbored the signal peptide and the HYDRO domain.

Gene expression analysis

Quantitative RT-PCR (qRT-PCR) was performed for 21 *S. luteus* and 17 *Heterobasidion* spp. genes. In Tables S3A-B the fold change was shown for genes that were significantly and not significantly differentially expressed.

Considering *S. lutues* interface, the most up-regulated gene was *SlGH18_673588* (fold 11.71) in dual culture with *H. irregulare* MUT00001193, while the most down-regulated gene was *SlHyd_804369* (fold 0.09) in dual

culture with *H. annosum* MUT00001204. Hierarchal clustering analysis on *S. luteus* gene expression in the interface areas allowed to identify three clusters (Fig. 5). The first cluster included genes not differently expressed in the different combinations, the second comprised significantly down-regulated genes, while the up-regulated genes were grouped in the last. Each cluster contained genes of different categories. In particular, there was a trend for lower expression in hydrophobins and a higher expression for chitinases.

In the outside area of *S. luteus* the most up-regulated gene was *SlHyd_14989* (fold 264.69) in dual culture with *H. annosum* MUT00001204, while the most down-regulated was *SlHyd_804369* (fold 0.14) in dual culture with *H. annosum* MUT00001204. Moreover, also in these areas, the same trend described above for the interface was observed.

Expression values of *Heterobasidion* spp. genes ranged from 0.0 to 10.73 in the interface area. In general *Heterobasidion* spp. genes showed a wide down-regulation in all the categories.

In the outside area of *Heterobasidion* spp. the expression range was between 0.04 and 22.79, corresponding to *HiHyd_65822* in *H. irregulare* MUT00001151 and to *HiGH20_306181* in *H. annosum* 137-OC 142-OH, respectively. Based on the HeatMaps (Fig. 5), a trend was observed for the expression of some genes, including three GH18, one GH20 and one hydrophobin, which showed a different regulation in the two species of *Heterobasidion* spp.

PCA did not allow to discriminate the two pathogenic species based both on their overall gene expression values during the interaction with the symbiont and on *S. luteus* expression values (Fig. 6).

However, in *S. luteus*, a gene coding for a chitinase (*SlGH18_8356*) was found to be significant differentially regulated in the outside area during interaction with *H. irregulare* compared to *H. annosum* ($df=4$, $p = 0.0012$). A *t* test confirmed that only the *Heterobasidion* spp. chitinase encoding gene *HiGH18_11431* was differentially regulated when the two pathogens were compared ($df=4$, $p = 0.0211$). In particular, a strong down-regulation for this gene was inferred in *H. irregulare* compared to *H. annosum*.

Discussion

It has been previously shown that ECM fungi may interact with soil and pathogenic fungi (Werner et al. 2002; Mucha et al. 2006; Mucha et al. 2009), but studies combining growth and molecular experiments to study the effects of non-native and native fungal pathogens on ECM fungi were still lacking. In this work, we have not only described the interaction between one isolate of *S. luteus* and three different isolates of the non-native *H. irregulare* and of the native *H. annosum*, but we have also assessed the gene expression during the

interaction. The use of different isolates of *Heterobasidion* spp. was due to the previously documented intra-specific genetic diversity (Werner and Lakomy 2002). The European isolate of *S. luteus* was employed not only for its geographic origin, but also for the availability of its genome, which has been recently sequenced by DOE Joint Genome Institute (JGI) in the frame of the Mycorrhizal Genomics Initiative (Kohler et al. 2015). In addition, host range of this ECM fungus is similar to that of *Heterobasidion* spp., encompassing several pine species (Dahlberg and Finlay 1999; Garbelotto and Gonthier 2013).

It is well known that the long term storage of pure cultures and sub-culturing may affect the genetic stability of fungal isolates, thus introducing possible biases when comparative analyses are performed (Thomson et al. 1993; Lalaymia et al. 2014). While we cannot exclude that such biases may have influenced our results, the maintenance methods used in the study are well-established and are routinely employed for both growth assays and gene expression analyses of *Heterobasidion* spp. and *S. luteus* (Giordano et al. 2014; Kohler et al. 2015).

The morphological observations and measurements of the cultures showed that *S. luteus* was considerably and significantly inhibited by all *Heterobasidion* spp. isolates, except by *H. annosum* 137OC-142OH.

Isolates of *H. annosum* were previously shown to overgrow *S. luteus* (Napierała-Filipiak and Werner 2000). In this study we showed that the same occurred for the invasive *H. irregulare*. The fitted MCMC GLMMs suggested that the LIGRs observed in *S. luteus* and *Heterobasidion* spp. depended on the isolates rather than on the species of *Heterobasidion* spp. growing in dual cultures. In fact, the DIC values of M1s were substantially similar to the M0s ones, indicating that the inclusion of the *Heterobasidion* spp. as fixed factor could not improve the models performances, despite the significance achieved by the β coefficient in the M1 of *S. luteus*. On the contrary, a large gap between DICs was observed comparing M2s to either M0s or M1s. The decrease of the DIC observed switching from M0s and M1s to M2s indicated that the isolate is an adequate explicative factor to interpret *S. luteus* and *Heterobasidion* spp. IGRs. The overwhelming effect of the isolate on the species in driving the LIGRs was also arguable from the visual inspection of the box plots representing the IGRs of *S. luteus* and *Heterobasidion* species for each dual culture. Starting from the results of the growth experiments obtained in this study, further experiments should be performed to investigate the morphology of the hyphae in the interaction zone between *S. luteus* and the two *Heterobasidion* species in dual culture at microscopic level.

Concerning gene expression analysis during dual culture, in *S. luteus SlGH85_813330,* chitinase (*SlGH18_673588, SlGH18_8356* and *SlGH18_805786*) and hydrophobin (*SlHyd_14989* and *SlHyd_804369*) genes were up- and down- regulated, while in *Heterobasidion* spp. one or two representatives for each

category were up- and down- regulated (*HiGH85_47693, HiGH20_306181, HiGH20_61259, HiGH18_11431 HiHyd_105914, HIHyd_65822)*. It has been already demonstrated in another biological system that gene expression is affected by the interaction between fungi (Iakovlev et al. 2004). Our results confirmed the rewiring of the transcriptional machinery of the ECM and the pathogenic fungi. In particular up- and down-regulation of genes encoding chitinolytic enzymes, such as putative N-acetylglucosaminidases (GH85 and GH20) and chitinases (GH18), might mirror a remodeling of cell wall chitin components. In the interface two GH85 genes, *SlGH85_813330* and *HiGH85_47693,* showed an up-regulation suggesting a role for their encoded enzymes. Enzymes classified as members of the GH85 family catalyze hydrolysis processes acting on asparagine-linked glycan of various glycoproteins and glycopeptides (Umekawa et al 2008) that could play a role in the cell-to-cell interaction (Bowman and Free 2006).

The two genes coding for GH20 in *S. luteus* were not significantly different from the control and the two GH20 in *Heterobasidion* spp. were down-regulated. The hydrolysis of terminal non-reducing N-acetylglucosamine residues from chitin, generally due to these enzymes (Slámová et al 2010), was probably a process not triggered by the dual culture.

On the other hand, the relative expression of GH18 genes displayed variable transcript profiles in the different dual cultures. In general, in *S. luteus* there was not a common expression profile for all chitinase genes, even when members within the same chitinase subgroup were considered. These differential expression profiles indicate an absence of a common induction/repression expression pattern inside the *S. luteus* GH18 family, suggesting that they may not have totally redundant roles, but rather they could have different functions. Phylogenetic analyses, showing the presence of *S. luteus* sequences in almost all clades of the two main subgroups, might support this scenario.

In the subgroup A-III the proliferation of *S. luteus* chitinases compared to the reduced presence of *Heterobasidion* spp. members of this family (4 *vs* 2) is interesting. High and low abundance of saprotrophic basidiomycetes, and pathogenic basidiomycetes, respectively in GH18 subgroup A-III may reflect different life-styles of the species (Karlsson and Stenlid 2008). In addition, it has been speculated about the involvement of A-III GH18s in fungal-fungal interactions in basidiomycetes (Karlsson and Stenlid 2008). In *Trichoderma* species, it has been demonstrated that most of these enzymes show overlapping functions with an involvement in both self- and non-self fungal cell wall degradation, suggesting that the activity of these enzymes is regulated by the substrate accessibility rather than speciation of individual chitinases (Gruber and Seidl-Seiboth 2012). Gene expression results obtained in this work suggest that *S. luteus* chitinases cannot be grouped on the basis of a specific role. Members inside the same subgroup showed in fact

383 different trends in several considered conditions. By contrast, the GH18 gene family members of the
384 *Heterobasidion* spp. showed a similar down-regulation trend during dual culture with the symbiotic fungus,
385 mostly considering the interface area, suggesting that they were not involved in the inhibition of *S. luteus*.
386 Statistical analysis on gene expression data showed that a *S. luteus* gene encoding a chitinase
387 (*SlGH18_8356*) was perceived differently by the two pathogenic species. As inferred by phylogenetic
388 analysis, this chitinase belonged to subgroup B-V, together with its paralogous SlGH18_8357. Members of
389 the B cluster are proposed to be involved in interspecific fungal interactions (Karlsson and Stenlid 2008).
390 *SlGH18_8357* was generally significantly up-regulated both in the interface and in the outside area,
391 irrespective to the pathogenic species present in the dual culture; on the contrary its paralogous
392 *SlGH18_8356* was generally down-regulated, specifically in the outside area during the interaction with *H.*
393 *irregulare*. It could be hypothesized that the lack of co-evolution between the invasive *H. irregulare* and the
394 symbiont might have affected the activity of SlGH18_8356.
395 The genes down-regulated in the interface, but up-regulated in the outside zone may suggest that the
396 signals diffusing from the interface could lead to a regulated expression of some key genes in other regions
397 of the mycelium, during non-self interaction as reported by Adomas et al. (2006) in the dual culture *P.*
398 *gigantea – H. parviporum*. The non-self interaction could affect the synthesis of hydrophobic metabolites,
399 such as hydrophobins, as previously suggested (Rayner et al 1994). Hydrophobins secretion could lock the
400 hyphal boundaries at the interface zone or during the formation of the barrage zone (Rayner et al. 1994). In
401 addition, changes in hyphal hydrophobicity could also help to protect the fungus from hydrolytic enzymes of
402 the other organisms (Chaffin et al. 1998). It is particular worth noting that four *S. luteus* sequences belonged
403 to a group including the *Schizophyllum commune* Fr. Hyd 4, which might play a role during morphogenesis
404 of fruiting body in this model fungus (Ohm et al. 2010).
405 We can conclude that the pathogens can modulate the growth of the symbiont and that the gene expression
406 of target genes changes both in the symbiont and in the pathogens when the fungi coexist. Despite it was
407 not possible to distinguish the effects of the invasive pathogen from that of the native one on the ECM
408 fungus from a wide gene expression perspective, a single *S. luteus* gene encoding a putative chitinase was
409 found to differentially perceive the two pathogens, thus showing a diverse expression trend.
410 It might be speculated that the inhibition effect is probably due to other genes/proteins that we have not
411 considered in this work (*e.g.,* genes involved in nutrient assimilation). To clarify this point a non-target
412 approach, such RNAseq, should be used as well as a metabolomics analysis. This study was carried out *in*
413 *vitro*, with the understanding that growth and gene expression in a host plant could be different; however, the

identification of regulated genes during the dual culture will help to better understand the interactions occurring in the natural environment itself. Our findings elucidate the effects of a pathogen on growth and expression of several candidate genes during the saprotrophic life phase of the symbiotic fungus. This comparative study in dual culture will be pivotal to decipher the effects during the symbiotic life stage of *S. luteus*. Large scale transcriptomic data of *S. luteus-Pinus sylvestris* ectomycorrhizae revealed that some cell wall related genes considered in this work were up-regulated during the symbiotic stage (Kohler et al. 2015). In our dual culture system, a regulation of these genes has been observed. Thus, it could be speculated that, in the presence of the plant, expression pattern of cell wall related genes might change in a system including fungal pathogens. Based on this hypothesis, an experiment including inoculation of several isolates of *H. irregulare* and *H. annosum* on *Pinus* seedlings colonized by *S. luteus* is needed. While it has been reported that ECM fungi may have a protective role against root pathogens, favoring the plant growth (Perrin 1990; Branzanti et al. 1999), little it is known on the impact that pathogens, and especially invasive ones, have on a mycorrhizal fungal species that coexist in the same environment.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Adomas A, Eklund M, Johansson M, Asiegbu FO (2006) Identification and analysis of differentially expressed cDNAs during nonself-competitive interaction between *Phlebiopsis gigantea* and *Heterobasidion parviporum.* FEMS Microbiol Ecol 57:26-39

Baccelli I, Gonthier P, Bernardi R (2015) Gene expression analyses reveal a relationship between conidiation and cerato-platanin in homokaryotic and heterokaryotic strains of the fungal plant pathogen *Heterobasidion irregulare.* Mycol Progr doi: 10.1007/s11557-015-1063-x

445 Berg A, Meyer R, Yu J (2004) Deviance information criterion for comparing stochastic volatility models, J Bus
446 Econ Stat 22:107-120
447
448 Bowman SM, Free SJ (2006) The structure and synthesis of the fungal cell wall. BioEssays 28:799-808
449
450 Branzanti MB, Rocca E, Pisi A (1999) Effect of ectomycorrhizal fungi on chestnut ink disease. Mycorrhiza
451 9:103-109
452
453 Cervinková H (1989) Mycorrhizae and control of root pathogen *Heterobasidion annosum*. Agric Ecosyst
454 Environ 28:55-58
455
456 Chaffin WL, López-Ribot JL, Casanova M, Gozalbo D, Martínez JP (1998) Cell wall and secreted proteins of
457 *Candida albicans*: identification, function, and expression. Microbiol Mol Biol Rev 62:130-80
458
459 Crawley MJ (2013) The R book– 2nd edition. Wiley J and Sons Ltd, Chichester, UK
460
461 Dahlberg A, Finlay RD (1999) *Suillus*. In Cairney JWG, Chambers SM (ed), Ectomycorrhizal Fungi: Key
462 Genera in Profile, Springer-Verlag, Berlin, Germany pp 33-64
463
464 Eslaminejad Parizi T, Ansaria M, Elaminejad T (2012) Evaluation of the potential of *Trichoderma viride* in the
465 control of fungal pathogens of Roselle (*Hibiscus sabdariffa* L.) *in vitro*. Microb Pathog 52:201-5
466
467 Fries N (1978) Basidiospore germination in some mycorrhiza-forming Hymenomycetes. Trans Br Mycol Soc
468 70:319-324
469
470 Garbelotto M, Linzer R, Nicolotti G, Gonthier P (2010) Comparing the influences of ecological and
471 evolutionary factors on the successful invasion of a fungal forest pathogen. Biol Invasions 12:943-957
472
473 Garbelotto M, Gonthier P (2013) Biology, epidemiology, and control of *Heterobasidion* species worldwide.
474 Annu Rev Phytopathol 51:39-59
475

476 Garbelotto M, Guglielmo F, Mascheretti S, Croucher PJP, Gonthier P (2013) Population genetic analyses
477 provide insights on the introduction pathway and spread patterns of the North American forest pathogen
478 *Heterobasidion irregulare* in Italy. Mol Ecol 22:4855-69
479
480 Giordano L, Gonthier P, Lione G, Capretti P, Garbelotto M (2014) The saprobic and fruiting abilities of the
481 exotic forest pathogen *Heterobasidion irregulare* may explain its invasiveness. Biol Invasions 16:803-814
482
483 Gonthier P, Garbelotto M, Varese GC, Nicolotti G (2001) Relative abundance and potential dispersal range
484 of intersterility groups of *Heterobasidion annosum* in pure and mixed forests. Can J Bot 79:1057-1065
485
486 Gonthier P, Warner R, Nicolotti G, Mazzaglia A, Garbelotto M (2004) Pathogen introduction as a collateral
487 effect of military activity. Mycol Res 108: 468-470
488
489 Gonthier P, Nicolotti G, Linzer R, Guglielmo F, Garbelotto M (2007) Invasion of European pine stands by a
490 North American forest pathogen and its hybridization with a native. Mol Ecol 16:1389-1400
491
492 Gonthier P, Garbelotto M (2011) Amplified fragment length polymorphism and sequence analyses reveal
493 massive gene introgression from the European fungal pathogen *Heterobasidion annosum* into its introduced
494 congener *H. irregulare*. Mol Ecol 20:2756-2770
495
496 Gonthier P, Lione G, Giordano L, Garbelotto M. (2012) The American forest pathogen *Heterobasidion*
497 *irregulare* colonizes unexpected habitats after its introduction in Italy. Ecol Appl 22:2135-43
498
499 Gruber S, Vaaje-Kolstad G, Matarese F, Lo´pez-Monde´jar R, Kubicek CP, Seidl-Seiboth V (2011) Analysis
500 of subgroup C of fungal chitinases containing chitin-binding and LysM modules in the mycoparasite
501 *Trichoderma atroviride*. Glycobiology 21:122-133
502
503 Gruber S, Seidl-Seiboth V (2012) Self *versus* non-self: fungal cell wall degradation in *Trichoderma*. Microbiol
504 158:26-34
505

506 Hadfield JD (2010) MCMC methods for multi-response Generalized Linear Mixed Models: The MCMCglmm
507 R Package. J Stat Softw 33:1-22
508
509 Holdenrieder O (1984) Untersuchingen zur biologischen Bekämpfung von *Heterobasidion annosum* an
510 Fichte (*Picea abies*) mit antagonistichen Pilzen. II. Interaktionstests auf Holz. Eur J Forest Pathol 14:137-153
511
512 Iakovlev A, Olson A, Elfstrand M, Stenlid J (2004) Differential gene expression during interactions between
513 *Heterobasidion annosum* and *Physisporinus sanguinolentus*. FEMS Microbiol Lett 241:79-85
514
515 Ismail Y, McCormick S, Hijri M (2011) A fungal symbiont of plant-roots modulates mycotoxin gene
516 expression in the pathogen *Fusarium sambucinum*. PLoS One. 6:e17990
517
518 Karlsson M, Stenlid J (2008) Comparative evolutionary histories of the fungal chitinase gene family reveal
519 non-random size expansions and contractions due to adaptive natural selection. Evol Bioinform 4:47-60
520
521 Keller RP**,** Geist J**,** Jeschke JM, Kühn I (2011) Invasive species in Europe: ecology, status, and policy. Env
522 Sci Eur 23**:**23
523
524 Kéry M (2010) Introduction to WinBUGS for Ecologists – A Bayesian Approach to Regression, ANOVA,
525 Mixed Models and Related Analysis. Academic Press – Elsevier, London, UK
526
527 Kohler A, Kuo A, Nagy LG, Morin E, Barry KW, Buscot F, Canback B, Choi C, Cichocki N, Clum A, Colpaert
528 J, Copeland A, Costa MD, Dore J, Floudas D, Gay G, Girlanda M, Henrissat B, Herrmann S, Hess J,
529 Hogberg N, Johansson T, Khouja HR, LaButti K, Lahrmann U, Levasseur A, Lindquist EA, Lipzen A,
530 Marmeisse R, Martino E, Murat C, Ngan CY, Nehls U, Plett JM, Pringle A, Ohm RA, Perotto S, Peter M,
531 Riley R, Rineau F, Ruytinx J, Salamov A, Shah F, Sun H, Tarkka M, Tritt A, Veneault-Fourrey C, Zuccaro A,

532 Tunlid A, Grigoriev IV, Hibbett DS, Martin F (2015) Convergent losses of decay mechanisms and rapid
533 turnover of symbiosis genes in mycorrhizal mutualists. Nat Genet 47:410-5

534

535 Lalaymia I, Cranenbrouck S, Declerck S (2014) Maintenance and preservation of ectomycorrhizal and
536 arbuscular mycorrhizal fungi. Mycorrhiza. 24:323-37

537

538 Lass-Flörl C, Nagl M, Speth C, Ulmer H, Dierich MP, Würzner R (2001) Studies of *in vitro* activities of
539 voriconazole and itraconazole against *Aspergillus* hyphae using viability staining. Antimicrob Agents
540 Chemother 45:124-128

541

542 Lei ZP, Jin JR, Wang CW (1995) Antagonism between ectomycorrhizal fungi and plant pathogens. In:
543 Brundett M, Dell B, Malajczuk M (ed) Mycorrhizas for plantation forestry in Asia. Proceedings of an
544 International Symposium and Workshop, 7–11 November 1994, Kaiping, China. Australian Centre for
545 International Agricultural Research, Proceedings No. 62, Canberra, Australia, pp 77-81

546

547 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR
548 and the 2(-Delta Delta C(T)) Method. Methods 25:402-8

549

550 Lövei GL (1997) Biodiversity: global change through invasion Nature 388:627-628

551

552 Millard PJ, Roth BL, Thi HP, Yue ST, Haugland RP (1997) Development of the FUN-1 family of fluorescent
553 probes for vacuole labeling and viability testing of yeasts. Appl Environ Microbiol 63:2897-2905

554

555 Mohan V, Nivea R, Menon S (2015) Evaluation of ectomycorrhizal fungi as potential bio-control agents
556 against selected plant pathogenic fungi. JAIR 3:408-412

Mucha J, Dahm H, Strzelczyk E, Werner A (2006) Synthesis of enzymes connected with mycoparasitism by ectomycorrhizal fungi. Arch Microbiol 185:69-77

Mucha J, Zadworny M, Werner A (2009) Cytoskeleton and mitochondrial morphology of saprotrophs and the pathogen *Heterobasidion annosum* in the presence of *Suillus bovinus* metabolites. Mycol Res 113:981-990

Napierała-Filipiak A, Werner A (2000) Antagonism of higher fungi to *Heterobasidion annosum* (Fr.) Bref. in laboratory conditions. Dendrobiology 45:65-81

Nicolotti G, Gonthier P, Varese GC (1999) Effectiveness of some biological and chemical treatments against *Heterobasidion annosum* on Norway spruce stumps. Eur J For Pathol 29:339-346

Ohm RA, de Jong JF, Lugones LG, Aerts A, Kothe E, Stajich JE, de Vries RP, Record E, Levasseur A, Baker SE, Bartholomew KA, Coutinho PM, Erdmann S, Fowler TJ, Gathman AC, Lombard V, Henrissat B, Knabe N, Kües U, Lilly WW, Lindquist E, Lucas S, Magnuson JK, Piumi F, Raudaskoski M, Salamov A, Schmutz J, Schwarze FW, vanKuyk PA, Horton JS, Grigoriev IV, Wösten HA (2010) Genome sequence of the model mushroom *Schizophyllum commune*. Nat Biotechnol 28:957-63

Olson A, Aerts A, Asiegbu F, Belbahri L, Bouzid O, Broberg A, Canbäck B, Coutinho PM, Cullen D, Dalman K, Deflorio G, van Diepen LT, Dunand C, Duplessis S, Durling M, Gonthier P, Grimwood J, Fossdal CG, Hansson D, Henrissat B, Hietala A, Himmelstrand K, Hoffmeister D, Högberg N, James TY, Karlsson M, Kohler A, Kües U, Lee YH, Lin YC, Lind M, Lindquist E, Lombard V, Lucas S, Lundén K, Morin E, Murat C, Park J, Raffaello T, Rouzé P, Salamov A, Schmutz J, Solheim H, Ståhlberg J, Vélëz H, de Vries RP, Wiebenga A, Woodward S, Yakovlev I, Garbelotto M, Martin F, Grigoriev IV, Stenlid J (2012) Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. New Phytol 194:1001-13

585 Perrin R (1990) Interactions between mycorrhizae and diseases caused by soil-borne fungi. Soil Use
586 Manage 6:189-195
587
588 Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from
589 transmembrane regions. Nat Methods 8:785-786
590
591 Pollastrini M, Luchi N, Michelozzi M, Gerosa G, Marzuoli R, Bussotti F, Capretti P (2015) Early physiological
592 responses of *Pinus pinea* L. seedlings infected by *Heterobasidion* sp.pl. in an ozone-enriched atmospheric
593 environment. Tree Physiol 35:331-40
594
595 Pysek P, Richarson DM (2010) Invasive species, environmental change and management, and health. Ann
596 Rev Environ Resour 35:25-55
597
598 Raffaello T, Asiegbu FO (2013) Evaluation of potential reference genes for use in gene expression studies in
599 the conifer pathogen (*Heterobasidion annosum*). Mol Biol Rep 40:4605-11
600
601 Raffaello T, Chen H, Kohler A, Asiegbu FO (2014) Transcriptomic profiles of *Heterobasidion annosum* under
602 abiotic stresses and during saprotrophic growth in bark, sapwood and heartwood. Environ Microbiol 16:1654-
603 67
604
605 Rayner AD, Griffith GS, Wildman HG (1994) Induction of metabolic and morphogenetic changes during
606 mycelial interactions among species of higher fungi. Biochem Soc Trans 22:389-94
607
608 Sivan A, Chet I (1989) Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. J Gen
609 Microbiol 135:675-682
610
611 Slámová K, Bojarová P, Petrásková L, Křen V (2010) β-N-Acetylhexosaminidase: What's in a name…?
612 Biotechnol Adv 28:682-693
613
614 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics
615 Analysis Version 6.0. Mol Biol Evol 30:2725-2729

Thomson BD, Malajczuk N, Grove TS, Hardy GESJ (1993) Improving the colonization capacity and effectiveness of ectomycorrhizal fungal cultures by association with a host plant and re-isolation. Mycol Res 97:839-844

Umekawa M, Huang W, Li B, Fujita K, Ashida H, Wang LX, Yamamoto K (2008) Mutants of *Mucor hiemalis* endo-beta-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities. J Biol Chem 283:4469-79

Werner A, Zadworny M, Idzikowska K (2002) Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture and the rhizosphere of *Pinus sylvestris* grown *in vitro*. Mycorrhiza 12:139-145

Werner A, Lakomy P (2002) Intraspecific variation in *Heterobasidion annosum* for mortality rate on *Pinus sylvestris* and *Picea abies* seedlings grown in pure culture. Mycologia 94:856-61

Werner A, Zadworny M (2003) *In vitro* evidence of mycoparasitism of the ectomycorrhizal fungus *Laccaria laccata* against *Mucor hiemalis* in the rhizosphere of *Pinus sylvestris*. Mycorrhiza 13:41-47

Wösten HAB, Scholtmeijer K (2015) Applications of hydrophobins: current state and perspectives. Appl Microbiol Biotech 99:1587-1597

Yakovlev I, Vaaje-Kolstad G, Hietala AM, Stefańczyk E, Solheim H, Fossdal CG (2012) Substrate-specific transcription of the enigmatic GH61 family of the pathogenic white-rot fungus *Heterobasidion irregulare* during growth on lignocellulose. Appl Microbiol Biotechnol 95:979-90

Zadworny M, Werner A, Idzikowska K (2004) Behaviour of the hyphae of *Laccaria laccata* in the presence of *Trichoderma harzianum in vitro*. Mycorrhiza 14:401-405

Supplementary material

Table S1A list of the *S. luteus* primers used in the current work and relative Temperature of annealing (Ta)

Table S1B list of the *Heterobasidion* spp. primers used in the current work and relative Temperature of annealing (Ta)

Table S2 MCMC GLMMs fitted on the inhibition growth rates of *S. luteus* and *Heterobasidion* spp. in logit scale (LIGR)

For each model (M0, M1, M2, M3), the intercept, the fixed and random factors (when included) are indicated. Under the factor "species", the coding level associated to *H. irregulare* and *H. annosum* is reported. The β and Z coefficients, for fixed and random factors respectively, with the associated 95% confidence interval ($CI_{95\%}$) and p-value (P_{MCMC}) are specified. The symbol * is associated to significant coefficients ($P < 0.05$).

Table S3A expression values in qRT-PCR of *S. luteus* genes

Table S3B expression values in qRT-PCR of *Heterobasidion* spp. genes

663

664 **Table 1** Fungal isolates used in the present work

665

Isolate code	Isolation date	Geographic origin	Species	MUT accession N.
39NE	2005	Castelfusano, RM, Italy	*H. irregulare*	MUT00001193
45SE	2005	Sabaudia, LT, Italy	*H. irregulare*	MUT00001151
CP15	2002	Castelporziano, RM, Italy	*H. irregulare*	MUT00003560
137OC-142OH	2013	artificial heterokaryotic isolate	*H. annosum*	XXX
Ha. Carp.	2007	Sabaudia, LT, Italy	*H. annosum*	MUT00001143
43NA	2005	Sabaudia, LT, Italy	*H. annosum*	MUT00001204
LMSL8	2009	Lommel Sahara, Limburg, Belgium	*S. luteus*	XXX

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667 *MUT Mycotheca Universitatis Taurinensis*

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672 **Figure Legends**

673 **Fig. 1** Schematic illustration of the experimental design for sample collection

674 **a***: S. luteus* pure culture, where the sampling point is in light grey*.* **b***: H. annosum* MUT00001143 sampling
675 point in dark grey. **c**: dual culture of *S. luteus* and *H. annosum* MUT00001143; the plugs were placed at 3 cm
676 from the side of the Petri dish and 1.5 cm between them. Three sampling points were shown: the outside of
677 the pathogen, the interface and the outside of the symbiont

678 **Fig. 2** Box plots of the inhibition growth rate (IGR in %) of *S. luteus* (a) and *Heterobasidion* spp. (b) for each
679 dual culture (1: *H. irregulare* MUT00001193, 2: *H. irregulare* MUT00001151, 3: *H. irregulare* MUT00003560,
680 4: *H. annosum* 137OC-142OH, 5: *H. annosum* MUT00001143, 6: *H. annosum* MUT00001204)
681 The bounding rectangles of the box plots include the values lying between the 25^{th} and 75^{th} percentile, the
682 horizontal thick black line is the median IGR, the t-shaped lines outside the rectangles indicate the minimum
683 and maximum values, the circles are outliers.

684 **Fig. 3** Phylogenetic relationships between filamentous fungi based on aminoacid deduced sequences for the
685 representative chitinase family
686 The sequences were aligned using Muscle and the tree was constructed using the Neighbor Joining (NJ)
687 method. Numbers indicate bootstrap values, and are given only for >50%. Red triangles represented *H.*
688 *irregulare* sequences, while blue circles represented *S. luteus* sequences.

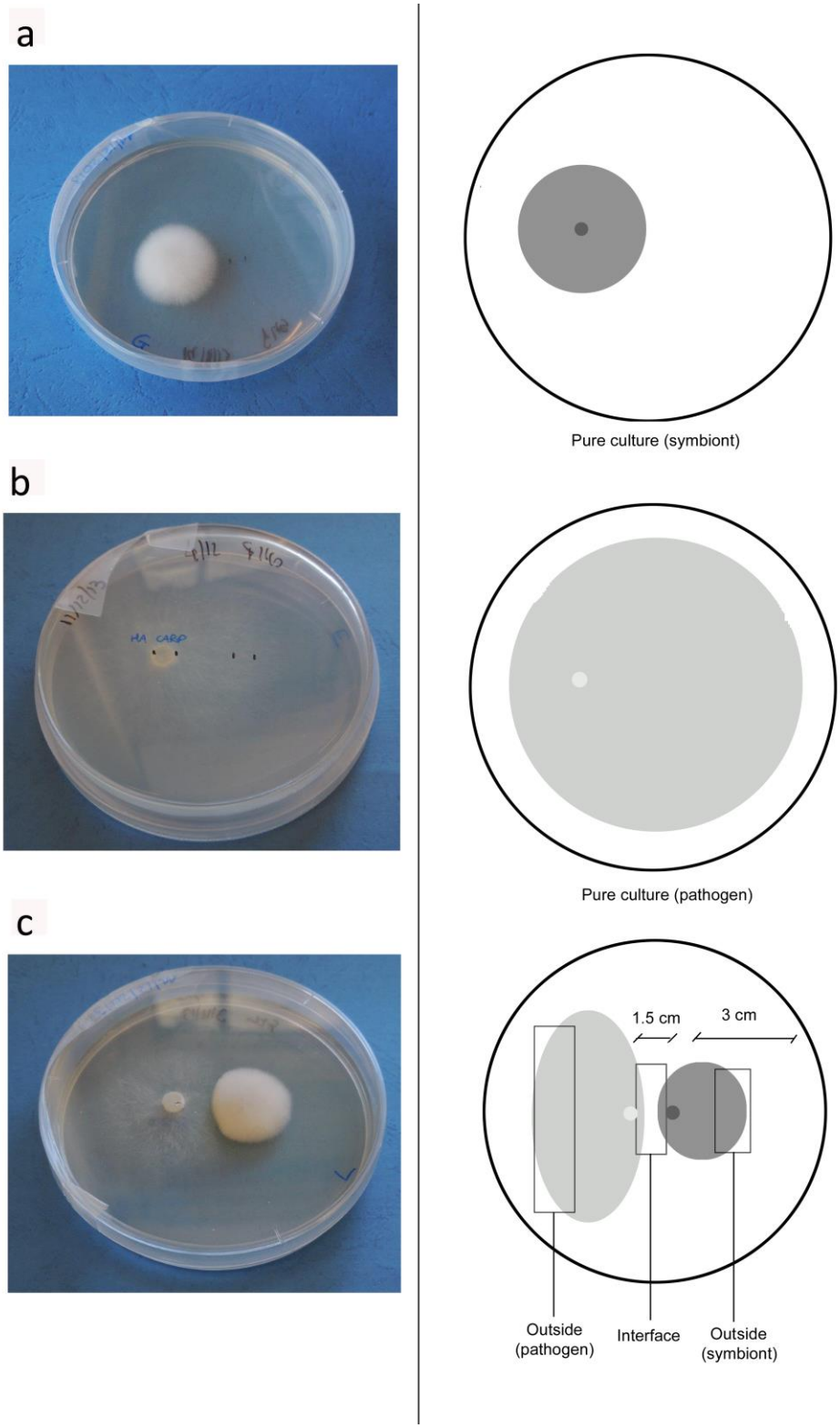
689 **Fig. 4** Phylogenetic relationships between filamentous fungi based on aminoacid deduced sequences for the
690 representative hydrophobin family
691 The sequences were aligned using Muscle and the tree was constructed using the Neighbor Joining (NJ)
692 method. Numbers indicate bootstrap values, and are given only for >50%. Red triangles represented *H.*
693 *irregulare* sequences, while blue circles represented *S. luteus* sequences.

694 **Fig. 5** HeatMap representation of the transcript levels coupled to a hierarchical clustering
695 The four HeatMaps consider the different sample areas for the gene expression analysis: *S. luteus* interface
696 (a), *S. luteus* outside (b), *Heterobasidion* spp. interface (c), *Heterobasidion* spp. outside (d). Each column
697 represents a fungal isolate (1: *H. irregulare* MUT00001193, 2: *H. irregulare* MUT00001151, 3: *H. irregulare*
698 MUT00003560, 4: *H. annosum* 137OC-142OH, 5: *H. annosum* MUT00001143, 6: *H. annosum*
699 MUT00001204), while each row represents a single gene. Expression levels are colored green for low
700 intensities and red for high intensities (see scale at the top right corner). The black cells represent genes not
701 significantly different from the control. The colors in the hierarchical clustering are: violet for GH18, blue for
702 hydrophobins, pink for GH20 and grey for GH85.

Fig. 6 Principal component analysis (PCA) of gene expression results in different combinations performed with MeV

The four plots considered the different sample areas for the gene expression analysis: *S. luteus* interface (a), *S. luteus* outside (b), *Heterobasidion* spp. interface (c), *Heterobasidion* spp. outside (d). White triangles represent combination of *S. luteus* and *H. irregulare*, while black triangles *S. luteus* and *H. annosum*.

709 **Fig. 1**



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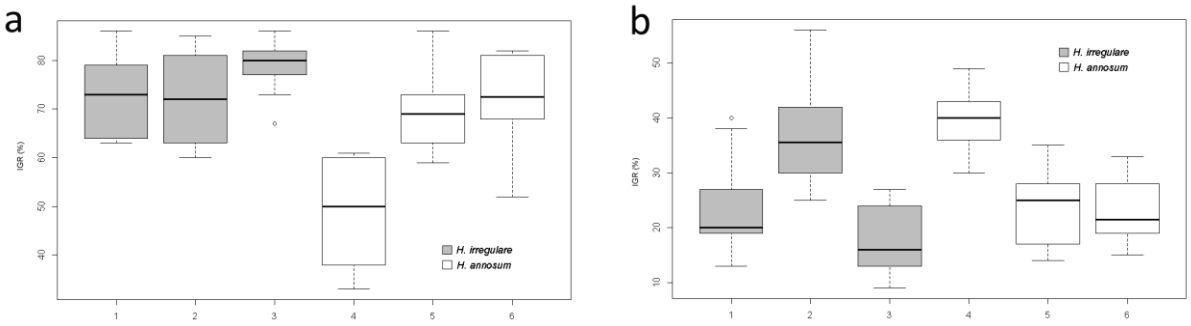
712 **Fig. 2**



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715 **Fig. 3**

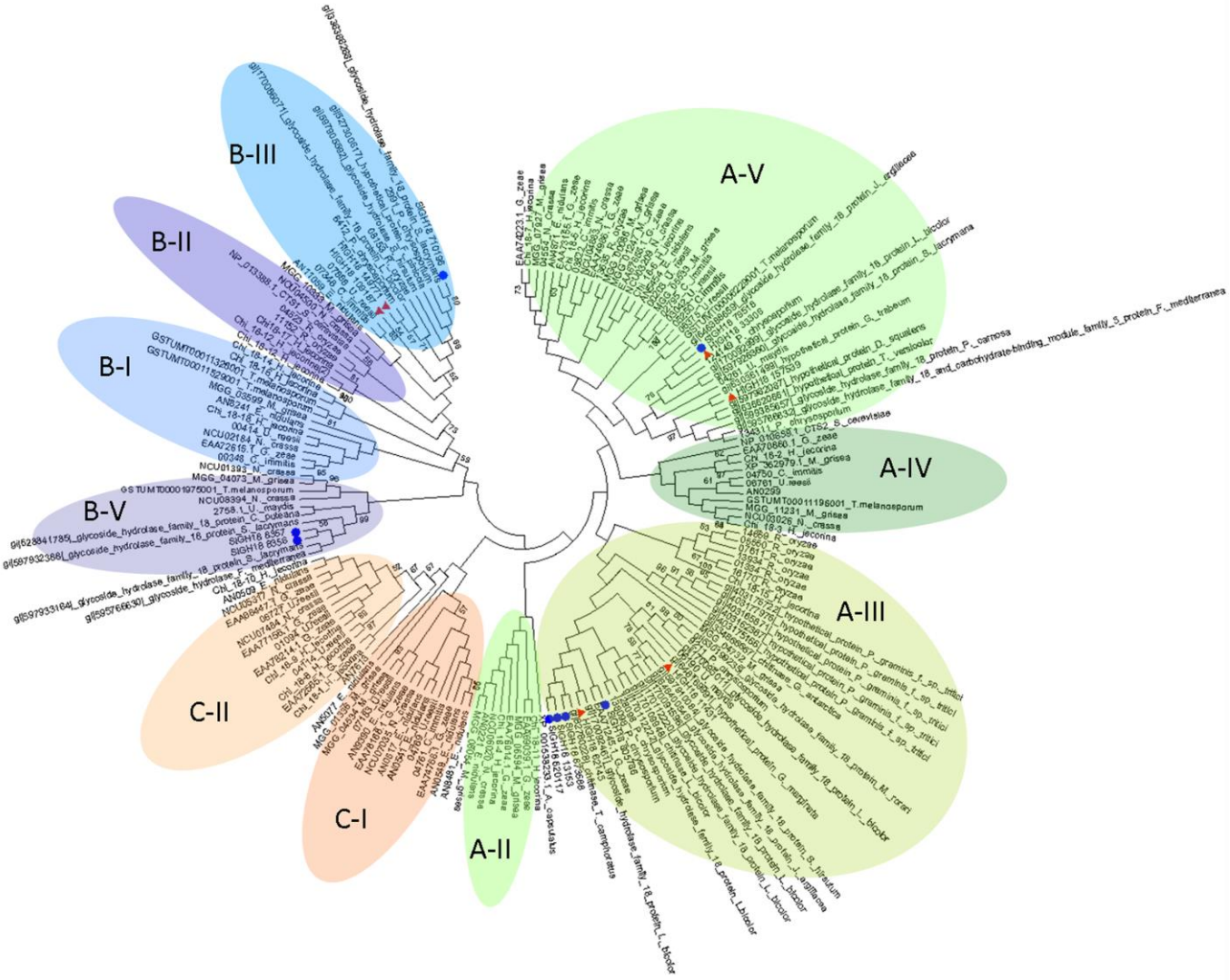

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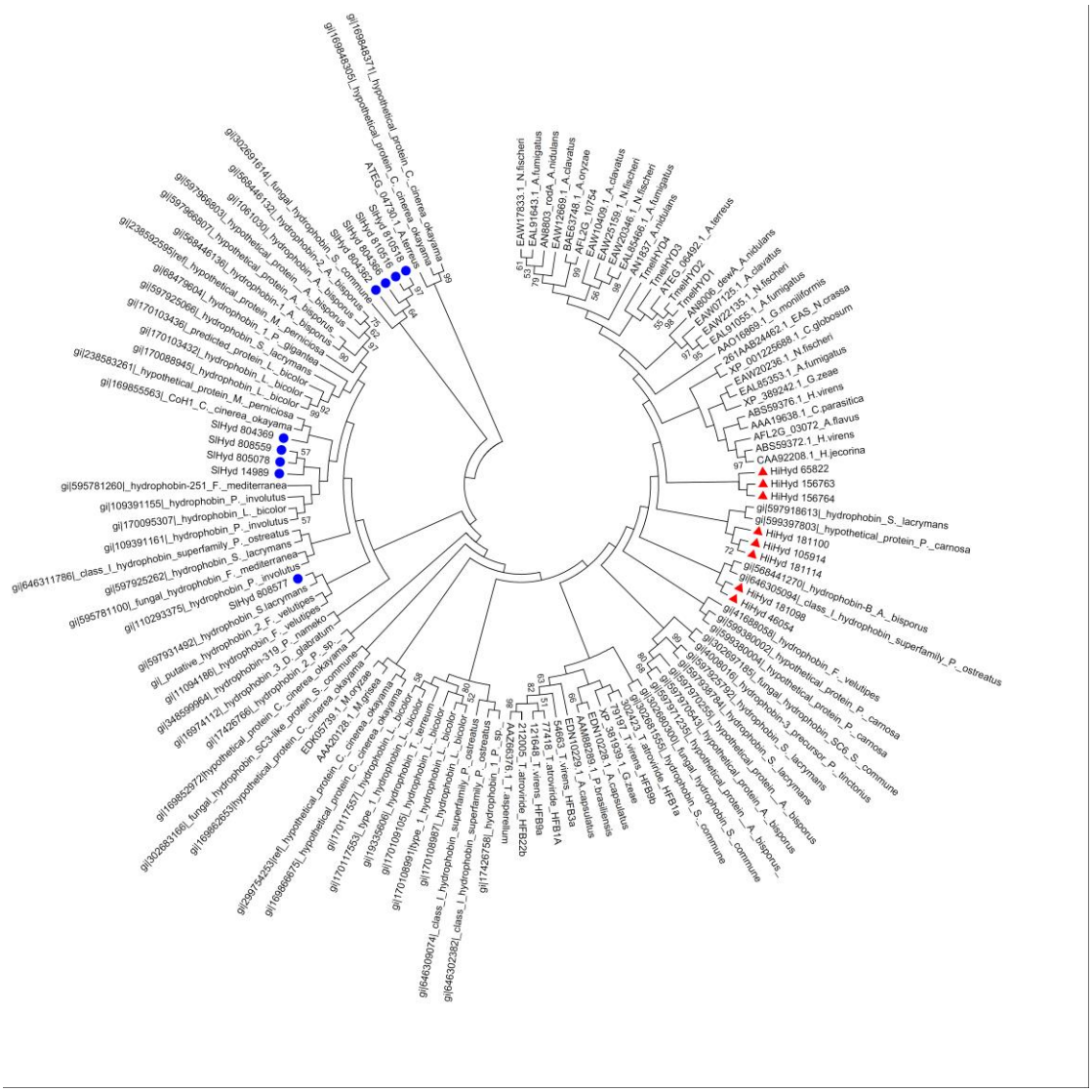
Fig. 4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SlHyd 804366
SlHyd 804362
gi|302891614|_fungal_hydrophobin_S._commune_
gi|56844613|_hydrophobin-2_A._bisporus_
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gi|56844613|_hydrophobin-7_A._bisporus_
gi|56479604|_hydrophobin_A._bisporus_
gi|597925066|_hydrophobin_M._perniciosa_
gi|170103436|_hydrophobin_1_P._gigantea_
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gi|169855563|_CoH1_C._cinerea_okayama_
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SlHyd 808559
SlHyd 805078
SlHyd 14989
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SlHyd 808577
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gi|_putative_hydrophobin_2_F._velutipes_
gi|110941186|_hydrophobin-319_P._nameko_
gi|348599964|_hydrophobin-3_D._glabratum_
gi|169741112|_hydrophobin_2_P._sp._
gi|174267586|_hydrophobin_C._cinerea_okayama_
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gi|302683166|_fungal_hydrophobin_SC3-like_protein_S._commune_
gi|169862653|hypothetical_protein_C._cinerea_okayama_
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AAA20128.1_M.grisea
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12164B_T.virens_HFB9a
77418_T.atroviride_HFB1A
54663_T.virens_HFB3a
EDN10229.1_T.P.brasilianis
AAN88289.1_A.capsulatus
XP_391039.1_G.zeae
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302023_T.atroviride_HFB1a
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BAE63748.1_A.oryzae_
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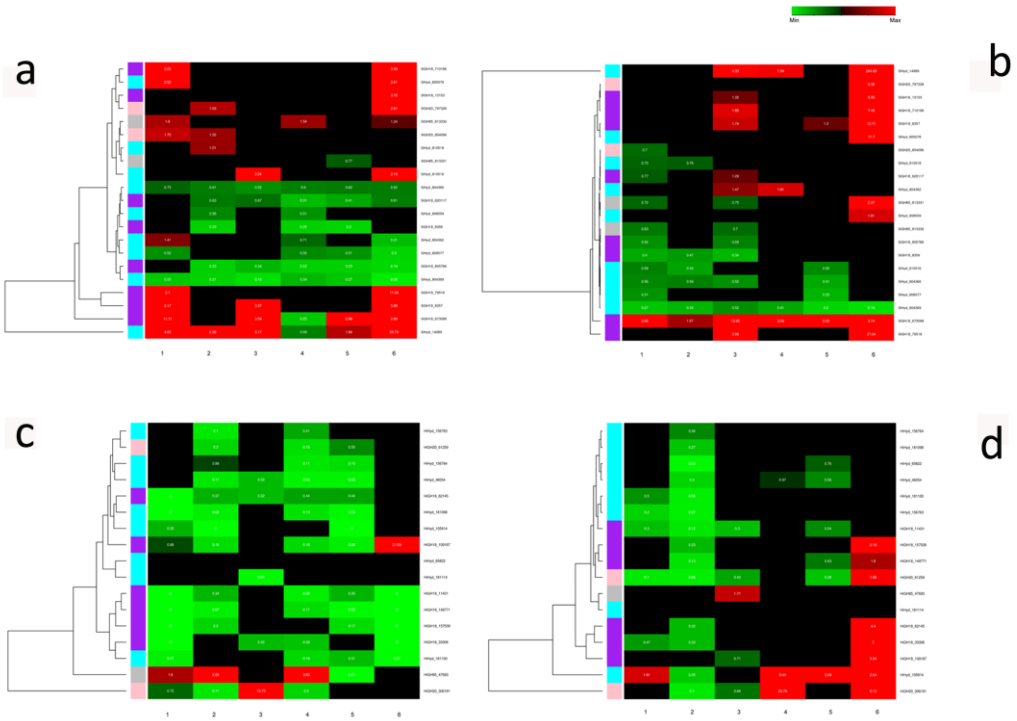
722 **Fig. 5**



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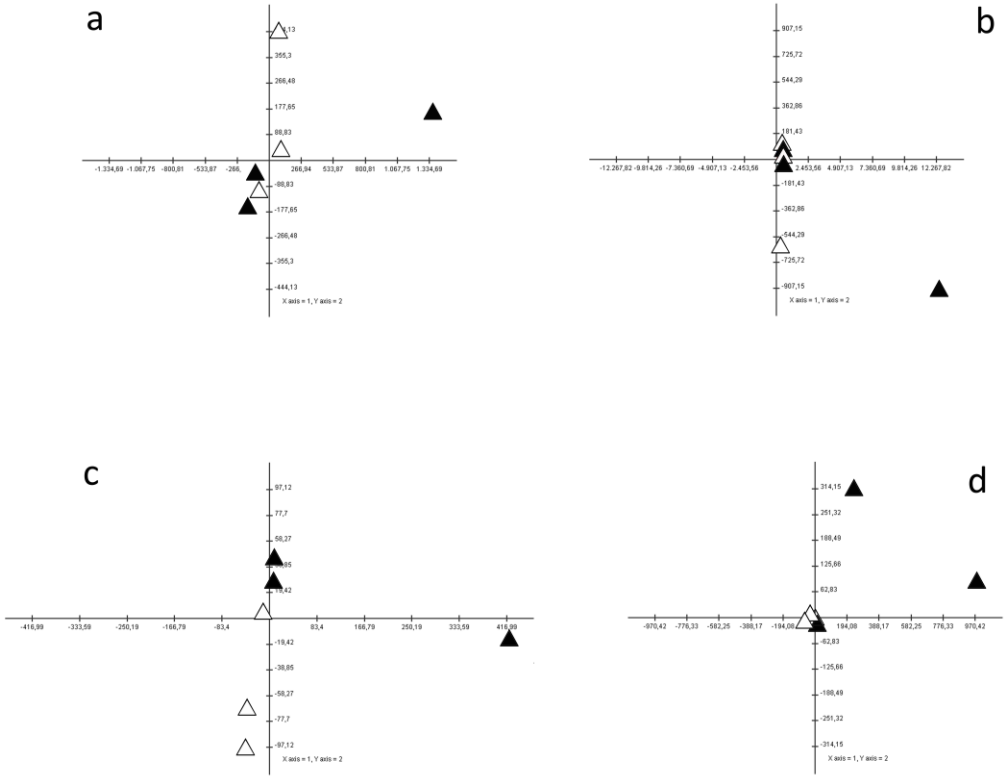
726 **Fig. 6**



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